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Two new species of *Lignosus* (*Polyporaceae*) from Malaysia — *L. tigris* and *L. cameronensis*

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Abstract —*Lignosus cameronensis* and *L. tigris* are described as new based on collections from the tropical forests of Pahang, Malaysia. Phenotypic and genotypic data support both as new species. Morphological features are illustrated, and the associated Internal Transcribed Region (ITS1 + 5.8S + ITS2) rDNA sequences have been deposited in the GenBank. Pore and basidiospore sizes are the main characters distinguishing these two *Lignosus* species from other members of the genus, with *L. tigris* distinguished from *L. sacer* by its larger pore size and smaller basidiospores and *L. cameronensis* separated from *L. ekombitii* by its smaller basidiospores. A key to the species of *Lignosus* is provided.

Key words — medicinal mushroom, sclerotia, taxonomy, phylogeny

Introduction

Lignosus Lloyd ex Torrend, a genus including six poroid species—*L. dimiticus* Ryvarden, *L. ekombitii* Douanla-Meli, *L. goetzii* (Henn.) Ryvarden, *L. hainanensis* B.K. Cui, *L. rhinocerotis* (Cooke) Ryvarden, *L. sacer* (Afzel. ex Fr.) Ryvarden—is known from the paleotropics (Cui et al. 2011, Dai 2012, Douanla-Meli & Langer 2003, Ryvarden & Johansen 1980). Members of this genus characteristically exhibit central stipitate basidiocarps, which rise from subterranean sclerotia. The genus is microscopically characterized by dimitic to trimitic hyphal systems, with dimitism currently reported only in *L. dimiticus,* which lacks binding hyphae in the context, sclerotium and stipe (Ryvarden & Johansen 1980). Apart from *L. goetzii,* basidiospores are known from all *Lignosus* species and are mostly hyaline, globose, isodiametric to ellipsoid depending on the species (Cui et al. 2010, Douanla-Meli & Langer 2003, Ryvarden & Johansen 1980).

Molecular analysis has supplemented conventional taxonomic classification of these macrofungi. Molecular markers used for *Lignosus* are the nuclear large subunit (nLSU), RNA polymerase II second largest subunit (RPB2), mitochondrial ATPase subunit six (ATP6) (Sotome et al. 2008), and the Internal Transcribed Region (ITS1 + 5.8S + ITS2) ribosomal DNA (Cui et al. 2010, Tan et al. 2010, Zhao et al. 2013, Tian et al. 2013). Easily amplified and possessing highly variable non-conserved ITS1 and ITS2 regions, the ITS region has proved useful for phylogenetic reconstruction of *Lignosus* species (Martin & Rygiewicz 2005, White et al. 1990).

Lignosus species have been widely studied due to their potential medicinal value. In Malaysia, locals regard the basidiocarps as important ingredients in traditional medicine, particularly for treating coughs and asthma, as well as for improving general well being (Chang & Lee 2004). Despite the promising benefits of these mushrooms, taxonomic research of *Lignosus* in Malaysia has been gradual and mediocre, with no significant records published since Ryvarden's discovery of *L. rhinocerotis* in the Penang island of Peninsular Malaysia (Ryvarden 1972).

Recent expeditions to the rainforests of Peninsular Malaysia have led to the discovery of two *Lignosus* species from the state of Pahang that conform to the characters of the genus but do not match any known species. We describe here these two new *Lignosus* based on morphological observations and molecular data and infer their phylogenetic relationships within *Lignosus*.

Materials & methods

Lignosus tigris specimens K and T were collected from a tropical forest in Lata Iskandar (4°17.46′N 101°34.41′E), Pahang, Malaysia on 12 June 2010; *L. cameronensis* specimens T1 and T8 were collected from the same forest on 17 July 2010. A small portion (50 mg) of sclerotia tissue was excised for maintaining a live culture and for DNA extraction; the remaining specimen was oven-dried for preservation as a herbarium specimen. *Lignosus tigris* K and *L. cameronensis* T1 herbarium specimens have been deposited at K(M) (Mycological Herbarium, Royal Botanic Gardens, Kew, UK); collections *L. tigris* T and *L. cameronensis* T8 are maintained in the personal herbarium of C.S. Tan, MARDI.

Morphological studies

Macroscopic characters are based on both fresh and subsequently dried material. Colors are based on the RGB color model, denoted by three numbers in brackets representing Red, Green, and Blue, respectively. Microscopic descriptions were carried out predominantly on dried specimens using a Nikon Z200 microscope with phase contrast illumination. Freehand sections were rehydrated in 70-95% ETOH prior to mounting in H_2O, 3% KOH, or Melzer's reagent. Basidiospores were observed and measured directly from the sections. Spore dimensions exclude the 5% extreme measurements from each end of the range given in parentheses. Q represents the mean length/width of the specified number of spores studied (n) (Largent 1977, Largent et al. 1977, Dai 2010).

Molecular taxonomy

DNA specimens were ground with fine sand powder, and DNA was extracted using a modified CTAB procedure (Cota-Sánchez et al. 2006). Polymerase Chain Reaction (PCR) amplification of the ITS was conducted in 25µl reaction volumes using the primers ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) and ITS5 (5′-GGAAGTAAAAGTCGTAACAAGG-3′) suggested by White et al. (1990). The PCR parameters were as follows: initial denaturing at 94°C for 5 min, followed by 20 cycles of (94°C for 45 sec, 45°C for 45 sec, 72°C for 1 min), and final extension at 72°C for 5 min. Amplicons were analyzed through staining with ethidium bromide and 1% agarose gel electrophoresis, and subsequently purified using a glass-milk matrix (Fermentas, USA) according to the manufacturer's protocol. Purified PCR products were ligated into pGEM-T Easy vector (Promega, USA) prior to transformation of the *Escherichia coli* strain JM109. Four clones were sequenced in both directions using forward and reversed M13 sequencing primers via 1st Base Laboratories Malaysia (ABI system).

The resulting DNA electropherograms of the ITS region were truncated and assembled into consensus sequences via ChromasPro V1.41 (Technelysium Pty Ltd). Multiple sequence alignments were generated with the incorporation of several sequences from the GenBank, and subsequently encoded in a NEXUS format using ClustalX V2.0 (Larkin et al. 2007). Details of samples used in this study are summarized in Table 1

Table 1. *Lignosus* and *Daedaleopsis* samples used in molecular analyses.

Sample	Locality	GenBank No.	DNA source
L. cameronensis T1 (holotype)	Malaysia	JQ409483	Sclerotium
T8 (isotype)	Malaysia	JQ409484	Sclerotium
L. ekombitii	Cameroon	(ex Douanla-Meli)	Sclerotium
L. hainanensis	China	GU580884	Sclerotium
L. rhinocerotis Ryvarden 11324	Malaysia	(ex Ryvarden)	Pileus
CH2	Malaysia	FJ380871	Sclerotium
CH31	Malaysia	FJ899143	Sclerotium
L. sacer Ryvarden 11329	Zimbabwe	GU001674	Pileus
K(M) 1037	Kenya	GU001675	Pileus
L. tigris K (holotype)	Malaysia	JQ409481	Sclerotium
T (isotype)	Malaysia	JQ409482	Sclerotium
D. confragosa (Bolton) J. Schröt	Germany	FR686551	—
D. sinensis (Lloyd) Y.C. Dai	—	FJ627256	—

Maximum parsimony (MP) and maximum likelihood (ML) analyses were used in the phylogeny construction via PAUP 4.0b10 (Swofford 2003). The MP tree was inferred through a heuristic search procedure using 1000 bootstrapping replications, with 100 random sequence addition and tree bisection reconnection (TBR) branch swapping. All characters were unordered and weighted equally. The best-fit HKY + G model was selected using hLRT ($\alpha = 0.01$) through Modeltest v3.7 (Posada & Crandall 1998), and implemented for the ML analysis. Parameters were similar to the MP analysis, but with 100 ML bootstrap replicates instead.

Bayesian inference (BI) was conducted using Mr. Bayes 3.2.1 (Huelsenback et al. 2001, Ronquist & Huelsenbeck 2003). Best-fit models were generated by Kakusan v.3 using the Bayesian Information Criterion (BIC) (Schwarz 1978) (Tanabe 2007). BI analysis

was performed using the Markov Chain Monte Carlo (MCMC) method over a run of 2,000,000 generations, with a tree sampled every 100 generations. Chain stationarity was determined graphically using Tracer v1.5 (http://tree.bio.ed.ac.uk/software/tracer/), and the first 20,000 trees were conservatively discarded as burn-ins prior to the construction of the final 50% majority rule consensus tree. The resulting phylogenetic inferences were analysed and processed via Figtree (Morariu et al. 2008).

Taxonomy

Lignosus tigris Chon S. Tan, **sp. nov.** PLATE 1

MYCOBANK MB 800843

Differs from *Lignosus sacer* by its larger pores and smaller basidiospores.

TYPE: Malaysia. Pahang, Lata Iskandar, 4°17.46′N 101°34.41′E, in tropical rain forests, 12 June 2010, Tan CS 'K' (**Holotype**, K(M) 177826; GenBank, JQ409481). Tan CS 'T' (**isotype**, MARDI; GenBank, JQ409482).

ETYMOLOGY: *tigris*, from the local folklore that the species arose from milk that dripped onto the ground while a tigress fed her cubs.

BASIDIOCARP annual, single, terrestrial, centrally stipitate with an isodiametric pileus; pilei up to 11.3 cm in diameter and 0.5 cm thick at the center; tough, without odor and taste. PILEUS obtusely umbonate, slightly depressed, concentrically zonate and cinnamon brown (169, 79, 5) to ochre (223, 161, 49), margin wrinkled, thin, slightly folded and becoming crisp when dry. Pore surface cream (249, 218, 161) to pale brown (234, 152, 4), pruinose when fresh. Pores roughly pentagonal or hexagonal, coherent. more or less honeycomb-like, 1–2 per mm, gradually smaller and denser towards the margin. Tubes layered, somewhat polygonal, apparent and deep, up to 4 mm depth; tube walls gradually thinning, becoming corky or crisp upon drying. Pileal context white (255, 245, 231), tough, up to 5 mm thick, turning cream (249, 218, 161) when dry. STIPE single, caramel brown (186, 81, 2), up to 14 cm long, 1–1.8 cm thick, slightly contorted; enlarged, rough and wrinkled at the base, gradually thinning and smoother towards the pileus. Context from stipe white (255, 249, 171), turning cream (249, 218, 161) when dry. SCLEROTIUM irregular, light-weight, wrinkled and soiled, up to 7 cm long and 6.5 cm broad. Context pure white (236, 236, 236) to cream (249, 218, 161), wrinkled, floccose-cottony to tomentulose around the core.

HYPHAL SYSTEM trimitic; all hyphae negative in Melzer's Reagent. Generative hyphae with abundant clamp, diameter less than 1.4 µm, hyaline and thin-walled, interwoven with flexuous, aseptate skeletal hyphae, 2.6–3.1 µm in diameter, within the hymenium. Binding hyphae common, branched, ranging from almost straight to tortuous, up to 2.3 µm in diameter. Trama composed predominantly of thick-walled skeletal hyphae, up to 3.4 µm diameter, with narrow lumen; and occasional binding hyphae, hyaline, branched, up to 1.9 µm.

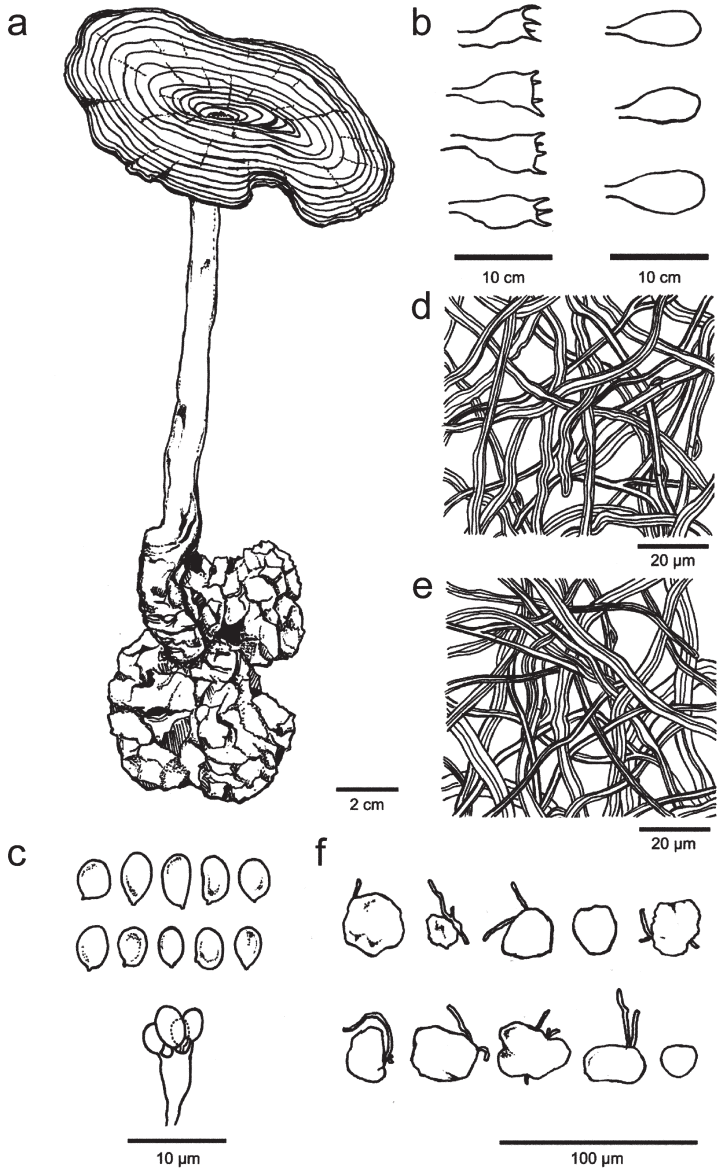

Plate 1. Microscopic structures of *Lignosus tigris* (holotype). **a** External morphology. **b** Basidia and basidioles. **c** Basidiospores and spore attachment. **d** Hyphae from trama. **e** Hyphae from stipe. **f** Sclerids.

Stipe context dominantly occupied by binding hyphae, hyaline, infrequently branched, diameter up to 1.9 µm; overlapped or slightly intertwined with sparse, slightly flexuous skeletal hyphae, hyaline, unbranched, diameter up to 4.7 µm, thick-walled with a narrow lumen. Sclerids dense and abundant within the sclerotium, subglobose, becoming somewhat oblong to irregular upon germination or when collectively clumped, 23.4–42.7 × 12.2–29.9 µm, margins wrinkled, appearing layered or stratified. Skeletal hyphae dominant, slightly sinuous, aseptate, hyaline, up to 3.5 µm in diameter, unbranched and thick-walled. Binding hyphae common, slightly flexuous, branched, interwoven, diameter up to 2.2 µm. Generative hyphae present, thin-walled, unbranched, hyaline, up to 1.5 µm in diameter.

Cystidia and cystidioles absent. Basidia clavate, subclavate, hyaline to granular, 4.9–9.1 × 1.75–3.3 µm; four sterigmata slender, generally 1.7–4.3 µm long, with some up to 4.3 µm long; basidioles mostly subclavate. Basidiospores mainly broadly ellipsoid to subglobose, infrequently globose and amygdaliform, (2.3–)2.5–5.5(–5.6) × (1.7–)1.8–3.6(–3.7) µm; Q = 1.34, (n = 44), thin-walled, hyaline, smooth.

Lignosus cameronensis Chon S. Tan, **sp. nov.** Plate 2

MycoBank MB 800844

Differs from *Lignosus ekombitii* by its smaller basidiospores.

Type: Malaysia. Pahang, Lata Iskandar, 4°17.46′N 101°34.41′E, in tropical rain forests, 17 July 2010, Tan CS 'T1' (**Holotype**, K(M) 177843, GenBank, JQ409483). Tan CS 'T8' (**isotype**, MARDI; GenBank, JQ409484).

Etymology: *cameronensis*, referring to Cameron Highlands, Malaysia; the locality of the type collection.

Basidiocarp annual, single, terrestrial, centrally stipitate with a more or less circular pileus; pilei up to 13.5 cm in diameter and 0.4 cm thick at the center; stout, without odor and taste. Pileus orbicular, slightly convex, brown (83, 32, 1) to chestnut brown (140, 55, 2), concentrically zonate, sulcate, rugose, margin deflexed, occasionally layered and overlapping; inrolled or incurved when dry. Pore surface cream brown (206, 157, 70), pruinose when fresh. Pores hardly visible, tiny and packed, isodiametric, 2–4 per mm. Tubes minute, dense, angular and layered; shallow, up to 2 mm deep; tube walls gradually thinning and becoming corky or crisp when dried. Tube layer up 4 mm thick. Context white (255, 245, 231), tough, up to 3 mm thick. Stipe muddy brown (175, 149, 97), single, central, equal, ellipsoidal, up to 8.5 cm long and 1.5 cm thick; woody hard and smooth when dry. Context from stipe cream (249, 218, 161). Sclerotium irregular, light, rugose and soiled, up to 6.5 cm long and wide. Context pure white (236, 236, 236), wrinkled and floccose-cottony around the center.

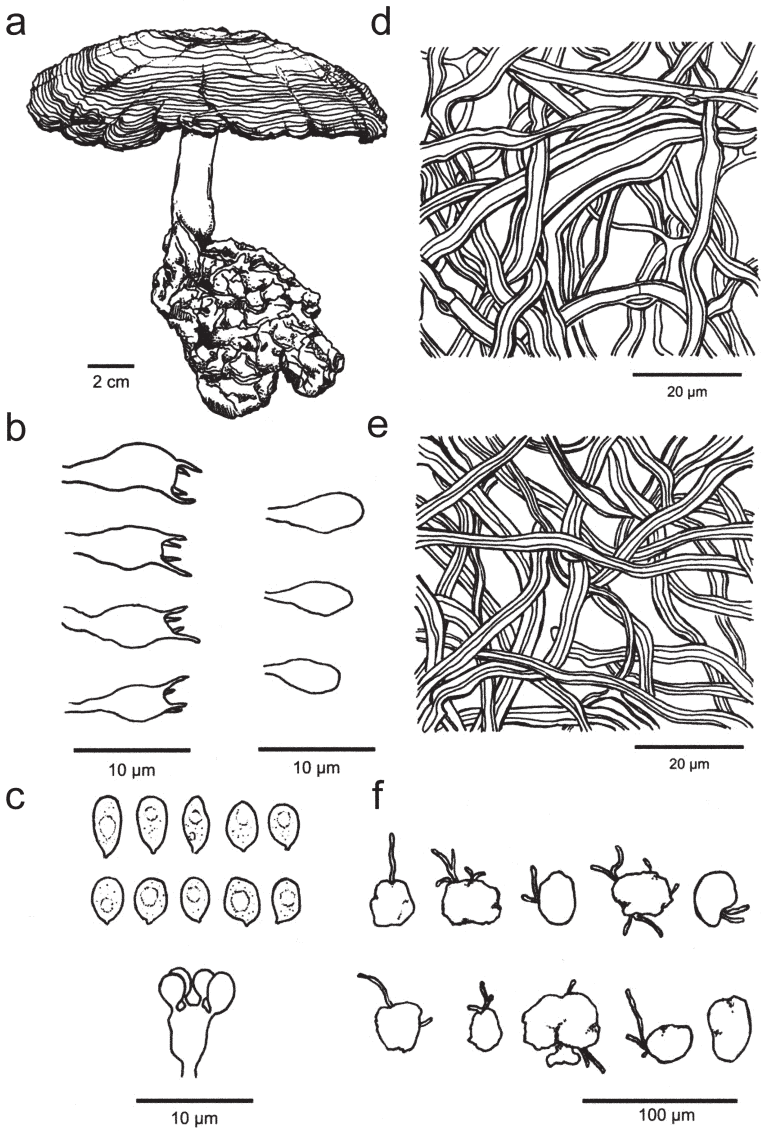

Plate 2. Microscopic structures of *Lignosus cameronensis* (holotype). **a** External morphology. **b** Basidia and basidioles. **c** Basidiospores and spore attachment. **d** Hyphae from trama. **e** Hyphae from stipe. **f** Sclerids.

Hyphal system trimitic; all hyphae negative in Melzer's Reagent. Generative hyphae with clamped connections, very irregular and contorted in the hymenium, thin-walled and hyaline, 1.2–1.5 µm in diameter; interwoven with occasional skeletal hyphae. Skeletal hyphae dominant within the trama, up to 3.6 µm in diameter, aseptate, thick-walled (up to 1 µm) with a narrow lumen, slightly flexuous, with scarce branching; mixed with thin-walled, almost straight to tortuous, aseptate binding hyphae, up to 2 µm in diameter; generative hyphae infrequent. Context of stipe, mainly of binding hyphae, integrating with relatively sparse, slightly flexuous skeletal hyphae; branching scarce. Sclerotium composed mainly of sclerids, originally subglobose or somewhat pear-shaped, becoming cordate to irregular upon germination or when clumped with other sclerids, 24.9–49.2 × 21.3–42.1 µm. Sclerid margins uneven, and seemingly stratified or striate. Skeletal hyphae dominant, thick-walled, hyaline, 1.3–4.1 µm in diameter, often branching. Generative hyphae less common, thin-walled, and unbranched, 1–1.6 µm in diameter. Binding hyphae present, interwoven, hyaline, often branching, 1.2–2.3 µm in diameter.

Cystidia and cystidioles absent. Basidia clavate, hyaline to granular, 3.6–8.9 × 1.4–4.5 µm; four sterigmata slender, generally 1.7–4.3 µm long, with some up to 5.7 µm long; basidioles mostly subclavate. Basidiospores mainly ellipsoid to subglobose, rarely globose, (2.2–)2.4–4.8(–4.9) × (1.8–)1.9–3.2(–3.4) µm; Q = 1.47, (n = 37), hyaline, smooth, thin-walled.

Molecular phylogeny

The ITS sequences were truncated at terminals to avoid ambiguous nucleotides, resulting in a length of 646 bp. Within the dataset, a total of 144 characters were considered parsimony-informative, whereas 466 characters remained constant. Parsimonious analyses of the ITS region resulted in two most parsimonious trees, with a consistency index (CI) of 0.902 and a retention index (RI) of 0.925. The nucleotide region encoding the 5.8S rRNA showed no variation among the *Lignosus* congeners. Conversely, large variations, including long indels were observed within the ITS1 and ITS2 regions. A genetic distance of up to 7.1% was observed between *L. tigris* and *L. cameronensis*. *Lignosus rhinocerotis* and *L. ekombitii* showed the highest genetic distance of up to 11.2% within the genus. An intraspecific genetic difference of 1.5% was observed between *L. cameronensis* T1 and T8. On the other hand, no intraspecific genetic variance was observed within the ITS region for the two *L. tigris* specimens.

The resulting phylogenetic tree (Plate 3) based on the ITS region indicates a clear distinction between the *Lignosus* ingroups and the *Daedaleopsis* outgroup. The suitability of this outgroup has been suggested and used in several studies (Cui et al. 2010; David & Michael 1995; Sotome et al. 2008). The monophyly (Clades A and B) of the *Lignosus* samples was inferred with high support

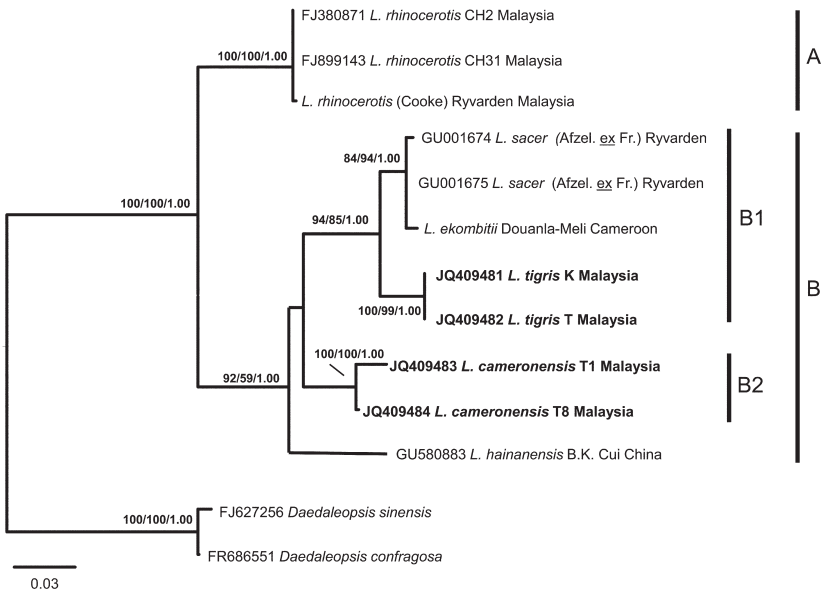

Plate 3. Maximum likelihood 50% majority-rule consensus tree based on the ITS region. Numeric values at nodes are arranged in the order of ML bootstrap support/MP bootstrap support/Bayesian posterior probabilities.

(ML = 100%; MP = 100%; BI = 1.00). The *L. rhinocerotis* sequences appeared as a sister group (Clade A) to the remaining *Lignosus* specimens. Clade B (ML = 92%; MP = 59%; BI = 1.00) is composed of *L. cameronensis* (Subclade B1); *L. tigris*, *L. sacer*, and *L. ekombitii* (Subclade B2); and *L. hainanensis*. The association between subclades B1 and B2 is currently unresolved. *Lignosus tigris* specimens (K and T) and *L. cameronensis* specimens (T1 and T8) each grouped with high support.

Discussion

Species currently placed in *Lignosus* generally have similar gross morphologies. Variations in size and dimensions of either sclerotia or basidiocarps are not characteristically unique to the species. Similarly, there is little variation between the hyphal systems, sclerids, etc. on a microscopic level. Currently, the sizes of the pores and basidiospores are the two characters sufficiently reliable to be used for species identification (Table 2). *Lignosus tigris* has strong similarities with *L. sacer*, and *L. cameronensis* with *L. ekombitii*.

Table 2. Pore and basidiospore sizes in *Lignosus*.

Species	Pores (per mm)	Basidiospores (µm)
L. goetzii	0.5–2	6–9 × 5–8
L. tigris	1–2	2.5–5.5 × 1.8–3.6
L. sacer	1–3	5–7 × 3–4.5
L. cameronensis	2–4	2.4–4.8 × 1.9–3.2
L. ekombitii	2–4	8.1–9.3 × 2.5–3.8
L. hainanensis	3–4	4.9–6 × 2.2–2.9
L. dimiticus	6–8	3–4.5 × 2.5–3
L. rhinocerotis	7–8	3–3.5 × 2.5–3

Lignosus tigris is differentiated from *L. sacer* by its larger pores (1–2 pores per mm) and smaller basidiospores (2.5–5.5 × 1.8–3.6 µm), and *L. cameronensis* differs from *L. ekombitii* by its smaller basidiospores (2.4–4.8 × 1.9–3.2 µm).

The phylogeny clearly separates *Lignosus rhinocerotis* from the other *Lignosus* species. All sampled *L. rhinocerotis* specimens clustered together with minor dissimilarities (<0.2% genetic distance), possibly resulting from spatial and time differences. The phylogenetic association between *L. cameronensis*, *L. sacer*, *L. ekombitii*, and *L. hainanensis* within Clade B remains unresolved with the ITS region molecular marker. This can eventually be addressed with longer gene regions, better molecular markers, and more specimens. The two *L. sacer* sequences (GenBank GU001674 and GU001675) differ slightly, with only two nucleotide substitutions at sites 69bp and 603 bp of the ITS region. The phylogenetic tree indicates that *L. ekombitii* is quite closely related, if not conspecific, with *L. sacer*; more specimens are needed to verify the identity of *L. ekombitii* phenotypically and genotypically. The high support for the monophylies of *L. tigris* and *L. cameronensis* confirms the morphological data that separate these from the other *Lignosus* species.

The molecular approach has thus far proved capable of inferring reliable phylogenetic relationships within *Lignosus*. More efforts in sequencing representatives of the remaining species and a broader sampling would help further elucidate this interesting genus.

Key to *Lignosus* species

1. Pileus white to ochraceous 2
1. Pileus light brown to dark brown 3

2. Pores large, 0.5–2 per mm ..... *L. goetzii*
2 Pores small, 6–8 per mm ..... *L. dimiticus*

3 Pores ≥7 per mm ..... *L. rhinocerotis*
3 Pores <6 per mm 4

4. Basidiospores broadly ellipsoid to subglobose 5
4. Basidiospores oblong ellipsoid to cylindrical 7

5. Basidiospores 5–7 µm long ..... *L. sacer*
5. Basidiospores 2.5–5.5 µm long 6

6. Pores 1–2 per mm ..... *L. tigris*
6. Pores 2–4 per mm ..... *L. cameronensis*

7. Basidiospores >6 µm long ..... *L. ekombitii*
7. Basidiospores <6 µm long ..... *L. hainanensis*

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